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Goat Milk Epithelial Cells Are Highly Permissive to CAEV Infection *in Vitro*

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The main route of small ruminant lentivirus dissemination is the ingestion of infected cells present in colostrum and milk from infected animals. However, whether only macrophages or other cell subtypes are involved in this transmission is unknown. We derived epithelial cell cultures, 100% cytokeratin positive, from milk of naturally infected and noninfected goats. One such culture, derived from a naturally infected goat, constitutively produced a high titer of virus in the absence of any cytopathic effect. The other cultures, negative for natural lentivirus infection, were tested for their susceptibility to infection with the CAEV-CO strain and a French field isolate CAEV-3112. We showed that milk epithelial cells are easily infected by either virus and produce viruses at titers as high as those obtained in permissive goat synovial membrane cells. The CAEV-CO strain replicated in milk epithelial cells in absence of any cytopathic effect, whereas the CAEV-3112 field isolate induced both cell fusion and cell lysis. Our results suggest that CAEV-infected milk epithelial cells of small ruminants may play an important role in virus transmission and pathogenesis.

INTRODUCTION

Until recently little attention was paid to a possible role of epithelial cells in lentivirus transmission and pathogenesis. Most human epithelial cells are $CD4^+$ and have therefore long been considered nonpermissive for HIV-1 infection, but these studies have been restimulated by the discovery that HIV-1 can infect cells by a CD4-independent mechanism (Yahi et *al*., 1992; Fantini et *al*., 1993). Replication of HIV-1 has been demonstrated in epithelial cells (Fantini *et al.,* 1993; Howell *et al.,* 1997) or cell lines (Fantini *et al.,* 1993; Tan et *al*., 1993) from different origins including the reproductive tract (Tan et *al*., 1993; Howell *et al.,* 1997), the gut (Fantini *et al.,* 1993), and the colon (Yahi *et al.,* 1992). This suggests that HIV-1 may cross the mucosal-epithelial barrier by direct infection of epithelial cells. The virus may also use a transcytosis pathway. In fact, it has been shown that HIV-1 can be transmitted to epithelial cells from lymphocytes or monocytes by a cell-to-cell contact mechanism (Phillips and Bourinbaiar, 1992; Bomsel, 1997). The virus is then transported from the apical to the basal membrane of epithelial cells using the transcellular vesicle pathway (Bomsel, 1997).

It has recently been reported that human mammary epithelial cells can be productively infected by HIV-1 (Toniolo *et al.,* 1995), suggesting that transmission of the virus through breast feeding may occur not only by infected lymphocytes/monocytes but also by virus-producing epithelial cells.

Small ruminant lentivirus (SRLV) infections of sheep and goats are worldwide and cause progressive degenerative inflammatory disease in multiple organs including the lung, joints, nervous system, and mammary gland (for review, see Cheblounе *et al.,* 1998). Several studies have shown that the main route of SRLV transmission is mucosal infection of newborn animals after ingestion of infected cells present in colostrum and milk from infected dams (Houwers, 1985; Kennedy-Stoskopf *et al.,* 1985; McGuire, 1987). Milking machines *are suspected to play* an important role in virus transmission through infected milk cells (Greenwood *et al.,* 1995). The small ruminant lentiviruses mainly target cells of the monocyte/macrophage lineage (Houwers, 1985; Kennedy-Stoskopf *et al.,* 1985; McGuire, 1987). The monocytes are infected but do not support virus replication until they differentiate into macrophages (Narayan *et al.,* 1982, 1983). However many other cell types, including epithelial cells from different organs, have been shown to harbor the viral genome *in vivo* (Zink *et al.,* 1990; Brodie *et al.,* 1995). In the lungs of sheep infected by maedi visna virus, the viral genome was found in a large number of bronchiolar epithelial cells (Staskus *et al.,* 1991).

Little is known of the sensitivity of epithelial cells to small ruminant lentivirus infection *in vitro*. In this study, our aim was to determine whether milk epithelial cells are permissive to caprine arthritis encephalitis virus

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(CAEV) infection *in vivo* or *in vitro*. To address this question, we derived milk epithelial cells from infected and uninfected nannies. From the infected goats, we isolated both naturally infected and noninfected epithelial cell cultures. The noninfected goats only furnished CAEV-negative cultures. Interestingly, however, the negative cultures were all highly susceptible to *in vitro* infection by either CAEV-CO or the French field isolate CAEV- 3112 and showed high permissivity for CAEV replication.

RESULTS

Development of epithelial cell cultures from milk

Milk epithelial cell cultures (MEC) were isolated from 100 ml milk from two infected and two noninfected goats. Tissue culture flasks (25 cm^2) were seeded with 2–5 $\times$ 10^6 cells, and after few days of culture the monolayers consisted mainly of fully differentiated mature macrophages. After 2–3 weeks, foci of epithelial cells, morphologically very distinguishable from macrophages, emerged and the macrophages progressively died. After two serial passages, homogenous epithelial cell culture monolayers were obtained. The great majority of the cells were cytokeratin marker positive in the early passages, and the later passage cells expressed the cytokeratin marker at 100% (Figs. 1a and 1b). The cells were positive only for cytokeratin; all cultures tested were negative when stained with antibodies directed against vimentin and actin (data not shown).

A naturally infected milk epithelial cell culture was derived from an infected goat

The MEC were tested for natural infection by immunocytochemistry using a monoclonal antibody specific for CAEV gag (McGuire, 1987) and one culture of four was positive (Figs. 1c–1e). Despite the expression of viral protein, no cytopathic effect was observed in this cell culture. However, on addition of goat synovial membrane (GSM) cells, giant multinucleated GSM cells appeared within a few hours, indicating that the envelope protein was correctly expressed on the epithelial cell surface. Radio-immunoprecipitation of viral proteins from both the supernatant and the lysate of this cell culture using a hyperimmune serum from a SRLV-infected goat indicated that all viral proteins were correctly expressed and processed (data not shown). The presence of Gag and Env proteins in the supernatant suggests that these cells actively produce virus particles. Supernatant from this epithelial cell culture was therefore harvested at different time points and virus was titrated according to the Reed and Muench method (Reed and Muench, 1938). Titers $\sim 10^{5,5}$ $TCID_{50}$ were obtained, indicating that the culture was chronically infected and constitutively produced a high titer of virus. Interestingly, a noninfected epithelial cell culture was obtained from one of the naturally infected goats.

In vitro infection of MEC with CAEV

Neither virus proteins nor infectious virus particles were detected in MEC cultures derived from the noninfected goats. We tested the susceptibility of these MEC cultures to CAEV infection after inoculation at m.o.i. of 0.1 either with CAEV-CO strain or with CAEV-3112 field isolate. Classical giant multinucleated cells (MGC) appeared 5–7 days after inoculation, and cell lysis was observed in the monolayers infected with CAEV-3112 (Figs. 2a and 2b). The cytopathic effects progressed, resulting in the progressive destruction of the cell monolayer. In contrast, infection with CAEV-CO strain produced no MGC or cell lysis (Fig. 2c). However, when CAEV-CO-infected MEC were cocultivated with GSM cells, spontaneous cell fusion was observed within 4–6 h (Fig. 2d).

The correct expression of viral proteins in CAEV-infected MEC was evaluated by radio-immunoprecipitation, which produced protein profiles similar to those observed after infection of GSM cells with CAEV-CO or CAEV-3112 (Figs. 3A and 3B). Little CAEV envelope glycoprotein was observed particularly in the cell lysate and supernatant of MEC infected with CAEV-CO. The p25 protein was detected in both the cell lysate and the supernatant of MEC infected by either CAEV-CO or CAEV-3112, suggesting active viral production. Supernatants of MEC infected by either CAEV-CO or CAEV-3112 were therefore harvested on Days 1, 3, 5, and 7 p.i., and viral titers were determined. All MEC cultures produced high titers of infectious cytopathic virus, and the kinetics of virus production were similar to those presented in the figure (Figs. 4A and 4B). CAEV-3112 showed the same kinetics on both GSM and MEC, but production of CAEV-CO on MEC was delayed as compared to GSM cells. This delay resulted in titers lower by 2–3 logs on Days 1–5 p.i. These low titers may be correlated to the low amount of envelope glycoprotein observed in the cell lysate and supernatant of MEC infected with CAEV-CO (Fig. 4). However, by Day 7, the titers on the two cell types were more similar. MEC produced a maximum titer of 10^5 $TCID_{50}$/ml for CAEV-CO and $>10^6$ and $TCID_{50}$/ml for CAEV-3112.

DISCUSSION

This study is the first report of productive CAEV infection by goat milk epithelial cells *in vitro*. The viral genome and/or proteins have been detected *in vivo* in epithelial cells from the lung, the thyroid, the kidney, and the small intestine of CAEV-infected goats (Zink *et al.,* 1990; Brodie *et al.,* 1995), but CAEV has not previously been shown to replicate in epithelial cells. The *ex vivo* culture of MEC derived from one of two naturally infected goats was chronically infected. Because macrophages were also present in the milk, we can not exclude the hypothesis that viral infection of the MEC occurred *in*

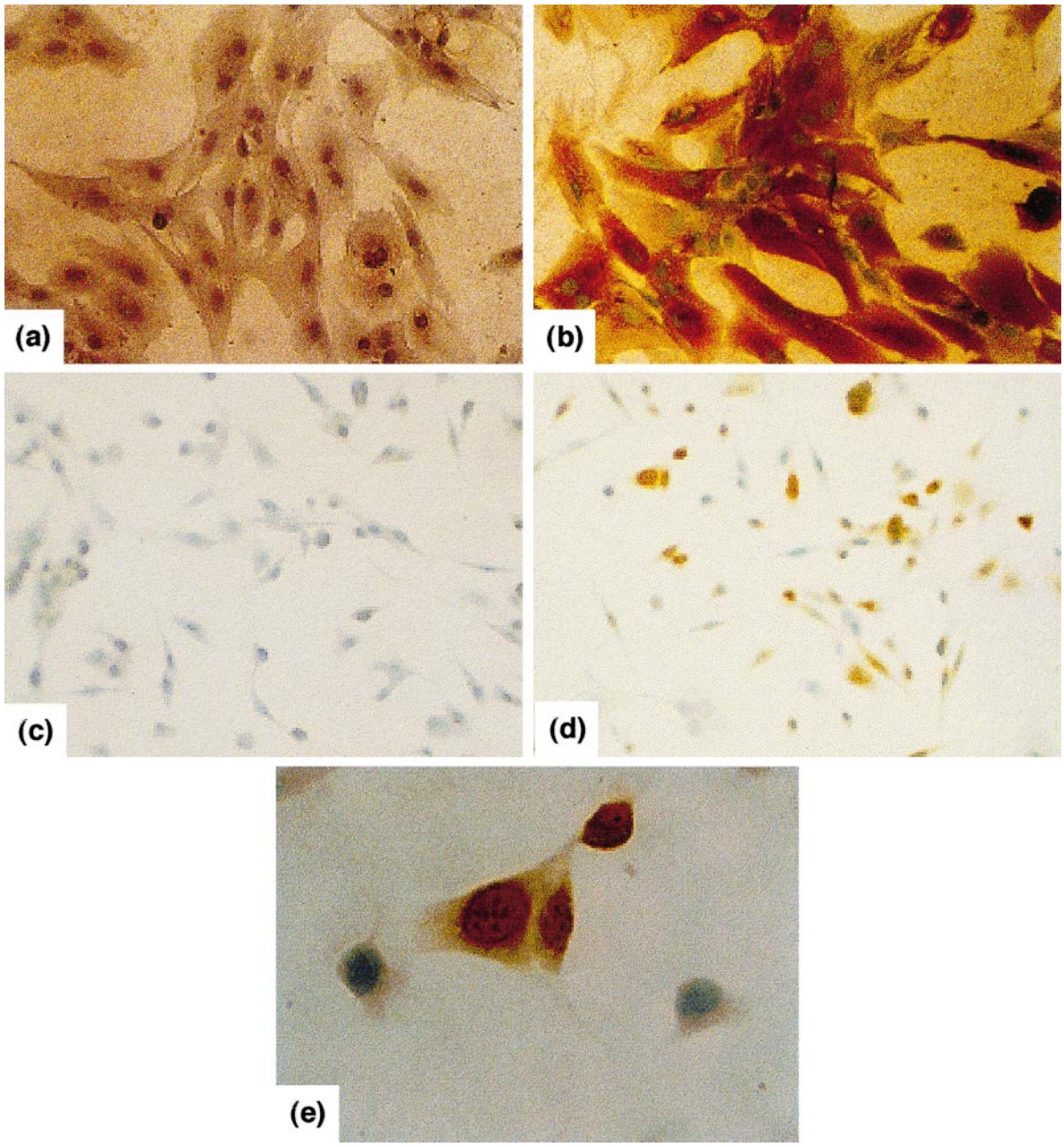

FIG. 1. Detection of cytokeratin and CAEV gag p25 protein expressions in cultured milk epithelial cells (MEC). (a and b) Immunocytochemistry was performed on MEC using the K813 clone cytokeratin specific antibody. The mock (a) corresponds to PBS-treated MEC and showed no positive cells. The MEC stained with the K813 monoclonal antibody (b) were 100% cytokeratin positive. (c–e) Immunocytochemistry was performed on MEC derived from naturally infected goats using an anti-p25 specific monoclonal antibody. Many cells express the CAEV p25 protein (d and e) as compared with the PBS-treated cells (c). Original magnification ×400 (a, b, and e) and ×100 (c and d).

vitro. However, this results demonstrated that MEC are sensitive to infection with a natural field virus and produced it at high titers. Virus production was constitutive and occurred in absence of any cytopathic effect in naturally infected cells and in those experimentally infected by one of two laboratory viruses. This response to lentiviral infection is not unique to goat epithelial cells because human epithelial cell lines derived from different organs have also been shown to produce HIV-1 in absence of cytopathic effect (Yahi *et al.,* 1992; Toniolo *et al.,* 1995). Isolation of naturally infected MEC from infected animals was not constant. From some infected animals we produced only noninfected cultures, and from the same animal the cultures produced were sometimes infected, sometimes not. However, the MEC culture we isolated were always sensitive to infection with both CAEV-CO and CAEV-3112 *in vitro*.

Our finding of high virus production from MEC may correlate with the high rate of CAEV transmission through milk that has been estimated as ≤85% (Rowe *et*

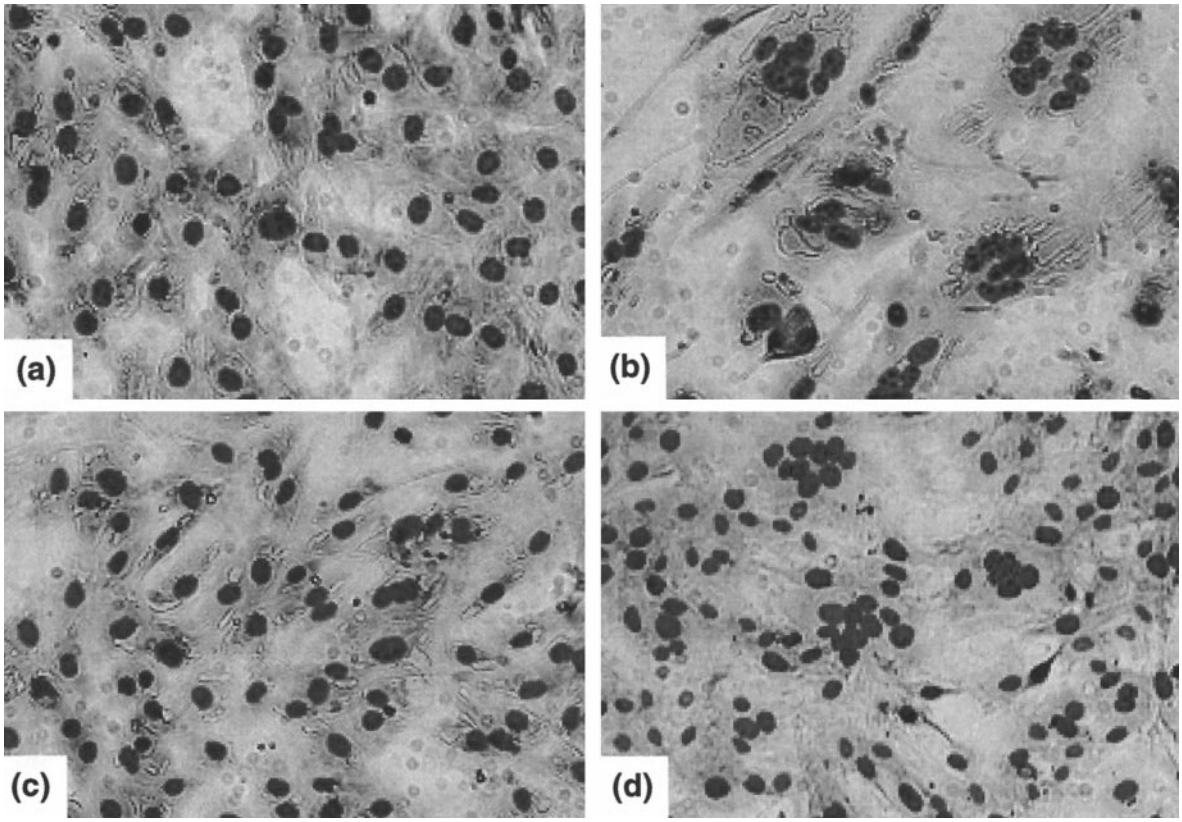

FIG. 2. Cytopathic effect developed in a milk epithelial cell (MEC) culture infected with two CAEV strains. (a) Uninfected MEC. (b) Typical giant multinucleated cell formation observed in MEC inoculated with CAEV 3112 field isolate. (c) No cytopathic effect was observed after inoculation of the MEC with CAEV-CO. (d) When CAEV-CO-infected MEC were cocultivated with goat membrane synovial cells (GSM), giant multinucleated cell formation was observed. Original magnification ×400.

al., 1992). Transmission is generally attributed to CAEV-infected macrophages, but little is known about the mechanism by which they might migrate across the intestinal epithelial barrier and infect blood cells in the recipient. Our results suggest that milk epithelial cells, or indeed the large number of free vesicular epithelial particles that may contain infectious virus, may also contribute to transmission by passing the virus on to intestinal epithelial cells. Viral RNA has been observed in goat intestinal crypt epithelial cells, suggesting possible transmission from infected milk macrophages or epithelial cells (Zink *et al.,* 1990). Transmission of CAEV may be more efficient when occurring between homologous cells, that is, MECs to intestinal epithelial cells. Lentiviruses passaged through different cell types show faster growth in the same cell type and display altered cell tropism (Cheng-Mayer *et al.,* 1991; Villarete *et al.,* 1994).

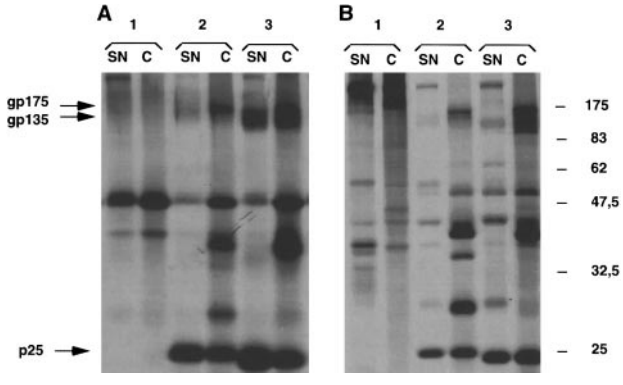

FIG. 3. Radio-immunoprecipitation of viral proteins from milk epithelial cell (MEC) cultures infected with two CAEV strains. The radio-immunoprecipitation was performed 6 days p.i. (A) Positive control corresponding to the immunoprecipitation of viral proteins on goat synovial membrane cells that were uninfected (1) or infected with CAEV-CO (2) or CAEV-3112 field isolate (3). SN, supernatant; C, cell lysate. (B) Detection of viral proteins on milk epithelial cells that were uninfected (1) or infected with CAEV-CO (2) or with CAEV-3112 field isolate (3).

The vigorous virus production by goat milk epithelial cells is unexpected given that infection of human epithelial cells with cell-free virus results in very low virus production (Tan *et al.,* 1993; Toniolo *et al.,* 1995). This difference may be due to poor infection efficiency of epithelial cells by HIV-1 using the galactosyl-ceramide rather than the CD4 receptor (Fantini *et al.,* 1993).

The high rate of replication of CAEV in MEC may also correlate with the high frequency of mastitis developing in CAEV-infected goats. Lesions develop in the mam-

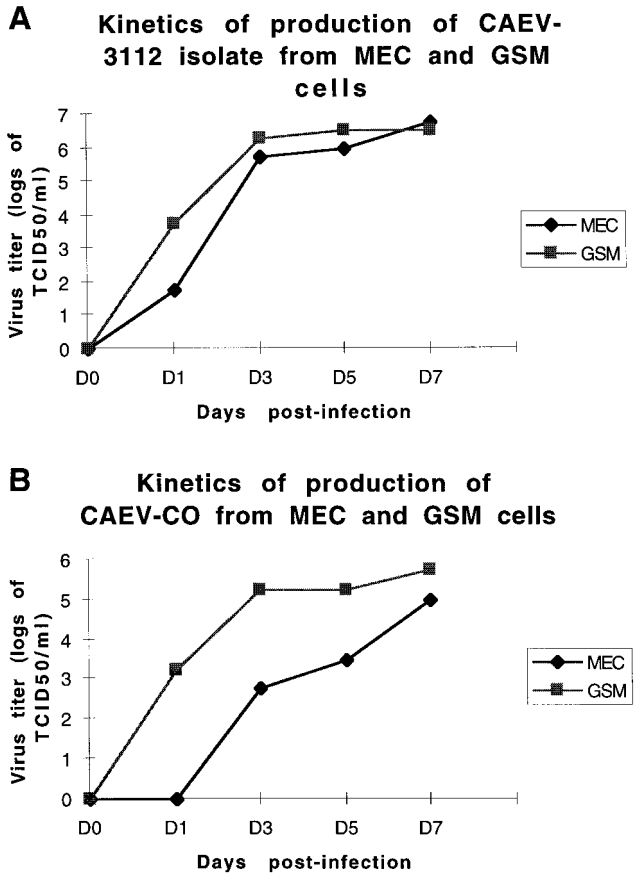

FIG. 4. Viral growth curves comparing production of CAEV-CO and CAEV-3112 on goat synovial membrane (GSM) cells and on milk epithelial cells (MEC). (A) Kinetic curve of production of CAEV-3112 field isolate on GSM and MEC. (B) Kinetic curve of production of CAEV-3112 on GSM cells and MEC.

mary glands of young goats fed with CAEV-infected colostrum and milk (Kennedy-Stroskopf *et al.,* 1985), and virus can easily be isolated from the mammary tissue of both kids and adult goats, demonstrating that the mammary gland is an important target organ for the CAE virus. Constitutive production of CAEV from goat mammary epithelial cells *in vivo* might trigger the extensive lymphocyte infiltration into the mammary gland of CAEV-infected goats.

Other retroviruses than HIV-1 may be transmitted through breast feeding. Luminal epithelial cells can be infected by BLV and may contribute to transmission (Buehring *et al.,* 1994). Persistent infection of breast luminal epithelial cells by HTLV-1 has been recently reported, suggesting that infection of this cell type may be involved in the persistence and transmission of HTLV-1, and explaining its endemic distribution in some ethnic groups (Southern and Southern, 1998).

Our data offer new insight into the sequence of events that may be involved in the transmission of virus to newborn subjects during milk feeding and in the induction of mastitis. These data may help to explain the high efficiency of transmission of CAEV by milk, which has led to the wide distribution of this virus throughout the world.

MATERIAL AND METHODS

Cells and viruses

Goat milk samples were obtained from four healthy yearling nannies 4 weeks postparturition. Two of them were naturally infected and two were not infected according to a CAEV serological test and virus isolation from blood-derived macrophages. The milk samples (100 ml) were centrifuged at 500 *g* for 10 min at +4°C, and cell pellets were rinsed twice with Hanks' balanced salt solution (HBSS) to remove the residual milk and fat globules. Cells were resuspended into 10 ml R10 (RPMI medium supplemented with 10% fetal bovine serum), seeded into 25 cm^2 tissue culture flasks at a density of 2–5 $\times$ 10^6 cells/ml and incubated at 37°C overnight. Nonadherent cells were flushed away by two to three rinses with HBSS, then fresh R10 medium was added and cells were incubated at 37°C in 5% CO_2. R10 medium was replenished every 3–4 days until subconfluent cell monolayers developed.

Goat synovial membrane cells (GSM) were obtained originally from explanted carpal synovial membranes from a colostrum-deprived newborn goat (Da Silva Teixeira *et al.,* 1997). GSM were expanded by cultivation in minimum essential medium (MEM) + 10% fetal bovine serum and stored in liquid nitrogen. Typical monolayer cultures were used for 7–10 passages.

The CAEV-CO virus was produced from GSM cells transfected with the pBSCA, a plasmid carrying the complete and infectious CAEV molecular clone (Mselli-Lakhal *et al.,* manuscript in preparation). Field virus isolate CAEV-3112 was obtained from a naturally infected French arthritic goat from the Lyon region (Blondin *et al.,* 1989). This virus was first produced by monolayer cultures of inflammatory cells aspirated from the synovium of the swollen joint and since passaged on GSM cell cultures. Stocks of both viruses produced on GSM cells or macrophages titered $\sim 10^6$ $TCID_{50}$/ml. Cells were infected 24 h after passage at a m.o.i. of 0.1, then 4 h after infection, the infectious supernatant was removed and replaced by fresh medium.

Virus titration

The supernatants of infected MEC or GSM cells were harvested and clarified by filtration through a 0.45-μm membrane. Serial 10-fold dilution in medium was used to infect GSM cells in 24-well plates, which were maintained in culture for 6 days. The cell monolayers were then Formalin fixed, stained with May-Grünwald-Giemsa, and examined for the presence of giant multinucleated cells. The titers were calculated using the Reed-Muench method (Reed and Muench, 1938) and expressed as

tissue culture infectious doses ($TCID_{50}$) per milliliter of supernatant.

Immunocytochemistry

Goat milk epithelial cells were seeded into eight-chamber slides (LabTek) and grown to subconfluence. Cytokeratin epithelial cell marker or viral proteins were detected on acetone-fixed cells by immunocytochemistry using specific monoclonal antibodies: clone K 813 (C6909, Sigma) for cytokeratin and anti-p25 CAEV (McGuire, 1987) for the detection of viral protein (30–5 A1, VMRD). Primary monoclonal antibodies were diluted in 1× PBS and 1% BSA (1/500 for anti-p25 and 1/50 for anticytokeratin) and added to cell monolayers. After 1 h of incubation at room temperature, cells were rinsed with PBS, then incubated for 30 min at room temperature in a solution containing 1× PBS, 1% BSA, and 0.5% of biotinylated goat anti-mouse Ig purified antibody (Dako Kit ref: K0377). Cells were rinsed with PBS and incubated for 30 min at room temperature in a 1× PBS solution containing streptavidin-biotin-peroxydase complex as recommended by the supplier. After a wash in 1× PBS, the cells were incubated for 8 min in 1× PBS solution supplemented with 1 mg/ml diaminobenzidine, rinsed with water, then counterstained with hematoxylin and mounted. Appropriate positive and negative controls were set up for each antibody.

Radio-immunoprecipitation of virus-specific proteins

Radio-immunoprecipitation of viral proteins was performed as previously described (Cheboulne *et al.,* 1996). Briefly, cells were seeded into six-well plates, and 24 h later, the monolayers were infected with CAEV-CO or CAEV-3112 at a m.o.i. of 0.1 and incubated at 37°C in 5% CO_2 for 4–5 days. The monolayer cultures were then pre-incubated for 2 h in MEM lacking methionin and cystein; the proteins then were radio-labeled for 16–18 h with 100 μCi of [^{35}S]methionin/cystein (ICN, Costa Mesh, CA) in 1 ml of the same medium. Virus-specific proteins released into the supernatant or present in the cell lysate fractions were immunoprecipitated using hyperimmune serum (G9615) from a goat that had received several injections of a mixture of three different CAEV and MVV-K1514 isolates. Clarified cell culture medium and cell lysates were incubated overnight at 4°C in the presence of 10 ml of G9615 serum and Sepharose protein A. Immunoprecipitated proteins were then separated by SDS–PAGE and specific virus proteins were visualized by standard autoradiography.

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